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Short communication

Capillary electrophoresis determination of thiopurine methyl transferase activity in erythrocytes

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ABSTRACT

Thiopurine S-methyltransferase (TPMT) catalyzes methylation of thiopurine drugs (e.g. 6-mercaptopurine, azathioprine). Decreased activity of TPMT is associated with hematopoietic toxicity after administration of standard doses of the drugs. We developed capillary electrophoretic method for determination of TPMT enzyme activity in erythrocytes. Limit of quantification of the method is 1.5 μmol/L (S/N = 6). The recovery of 6-methylmercaptopurine was 87.5–94.8%, imprecision value (as CV, $n = 10$) was 1.68% (within-day) and 2.53% (between-day). Erythrocyte TPMT activities were measured in 60 healthy adult volunteers.

1. Introduction

Thiopurine drugs (azathioprine, 6-mercaptopurine and 6-thioguanine) are widely used in both the treatment of acute lymphoblastic leukemia, autoimmune disorders and in immunosuppressive regimens for the prevention of acute rejection after solid organ transplantation. Active metabolites of these drugs, the 6-thioguanine nucleotides are considered to exert their therapeutic effect through incorporation into DNA with subsequent disruption of cell replication [1]. TPMT catalyzes conversion of thiopurines to non-toxic or less toxic methylated compounds, as opposed to their metabolic activation to cytotoxic 6-thiopurine nucleotides [2,3] and represents the key enzyme of thiopurine degradation metabolism. Approximately 89% of the Caucasian population are wild-type and have normal activity, 11% are heterozygous and have intermediate activity, and 0.3% are homozygous and have low activity [4–9]. Individuals with low or undetectable TPMT activity are at high risk for developing major and eventually life-threatening myelosuppression at standard doses of thiopurine drugs [10]. Patients with high TPMT activity may experience treatment failure [11] or hepatotoxicity, but the situation is much less clear than for the TPMT deficiencies [3].

First method for TPMT activity determination was based on the enzyme catalyzed transfer of the radioactive ^{14}C-labelled methyl group of S-adenosyl-L-methionine (SAM) to the thiol group of 6-mercaptopurine (6-MP) [12]. Subsequently, Lennard and Singleton [13] and Jacqz-Aigrain [14] introduced first non-radioactive HPLC methods for the determination of TPMT activity.

We have developed and validated a capillary electrophoretic method for screening of TPMT deficient patients in human erythrocytes using 6-MP as a substrate.

2. Experimental

2.1. Chemicals

All chemicals were of analytical-reagent grade except the chloride salt of SAM, which was 70%. Diethyl ether was obtained from Lach-Ner (Neratovice, Czech Republic). 6-Methylmercaptopurine (6-mMP) was obtained from Agros Organics (New Jersey, USA). Chloride salt of SAM, 6-MP, triethylamine and other chemicals were obtained from Sigma (St. Louis, MO, USA). 6-mMP and 6-MP were dissolved in dimethyl sulfoxide and deionized water (18.3 MΩ/cm) was used for the preparation of all other solutions. Buffer was prepared from 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and adjusted with triethylamine.

2.2. Preparation of the cell lysate

Blood samples from healthy blood donors were collected into EDTA tubes. All patients provided signed informed consent. Erythrocytes were separated from whole blood by centrifugation (1200 × *g*, 5 min). Plasma and buffy coat were removed and discarded. Erythrocytes were washed twice with 0.9% NaCl. Packed red blood cells (200 μL) were resuspended in 800 μL of ice-cold distilled water (this step results in cell lysis). The lysate was cen-

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trifuged (5000 × g, 10 min). The supernatant was used to measure the TPMT activity and hemoglobin content (Radiometer ABL 725, Diamond Diagnostics).

2.3. TPMT activity assay

TPMT activity was determined by measuring the 6-methylmercaptopurine (6-mMP) formed in the enzymatic reaction by the use of capillary electrophoresis. For enzymatic reaction 200 μL of supernatant of erythrocyte lysate was mixed with K_2HPO_4 (30 μL, 300 mmol/L, pH 7.5), allopurinol (16 μL, 1 mmol/L), dithiothreitol (6 μL, 50 mmol/L), SAM (4 μL, 2 mmol/L), and 6-MP (20 μL, 20 mmol/L) and incubated (2 h, 37 °C). After incubation, the samples were deproteinised with trichloracetic acid (50 μL, 1 mol/L), sonicated (30 s) a centrifuged (5000 × g, 1 min). The supernatant was immediately back-extracted (for 20 s while vortex mixing) twice with 1.5 mL of diethyl ether. Upper layer (containing diethylether with trichloracetic acid) was discarded and lower water fraction was left open for a 10 min in an eppendorf tube on bench (evaporation rest of diethylether). Sample was injected into the capillary or stored at −50 °C. Activity was calculated by dividing of measured concentration of mMP (μmol/L) and hemoglobin concentration in the lysate (g/L) and it was expressed as nanomoles of product formed per 1 h of incubation time.

2.4. Capillary electrophoresis

All experiments were performed on P/ACE 5510 with diode array detector (Beckman Instruments, Fullerton, USA). The electrophoretic separations were carried out in an uncoated fused-silica capillary (75 μm i.d. × 375 μm o.d.; Polymicro Technologies). The capillary had an effective length of 20 cm (total length, 27 cm) and was operated at 25 °C. Ultraviolet detection over the range 190–330 nm was used. For quantification of analytes sum of absorbances in the range of 284–304 nm was applied. The data rate of detector was set at 2 Hz for analyses performed at 20 kV. Samples were loaded by low-pressure injection (0.5 psi, 3 s, 13 nL of sample injected). Buffer was filtered (13 mm nylon syringe filter, 0.45 μm; Fisons Ins., Mountain View, CA, USA) and sonicated for 0.5 min before use. At the beginning of each working day, the capillary was washed by pressure (20 psi) with water and separation buffer for 5 min and between runs with 0.1 M sodium dodecylsulfate (SDS) for 1 min and separation buffer for 1 min.

2.5. Validation of the TPMT activity assay

The linearity of the method was assessed by used of erythrocyte lysate with 6-mMP added to yield concentrations between 2 and 200 μmol/L (eight different concentrations, $n = 5$ for each concentration). The recovery of the reaction product was measured using three additions of 6-mMP (final concentrations 5, 10, 20 μmol/L, $n = 3$ for each concentration) to erythrocyte lysate. The within-day and between-day imprecision were performed using erythrocyte lysates to which 6-mMP (final concentration 20 μmol/L) was added.

3. Results and discussion

3.1. Optimisation of capillary electrophoretic conditions

Optimisation experiments were performed using mixture of incubated samples from 10 erythrocyte separate lysate samples and using standard mixture of 6-mMP a 6-MP.

For initial experiments commonly used alkaline buffer consisting of 80 mmol/L borate (pK = 9.2) titrated by 2-amino-2-methyl-1-propanol (pK = 9.7) to pH 8.7–9.7 was chosen for separation. Under these conditions separation of standard mixture was obtained, but

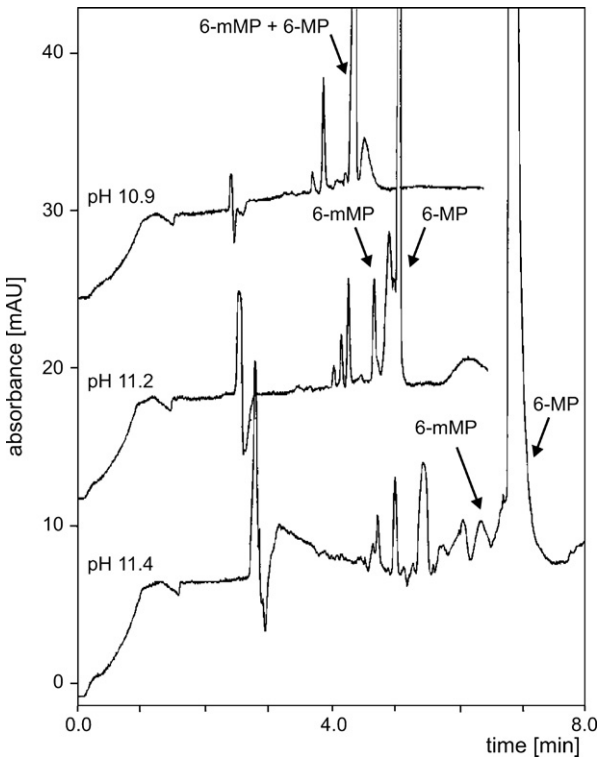

Fig. 1. Analysis of pooled incubation mixture in buffer in the pH range of 10.9–11.4; conditions: 80 mmol/L CAPS titrated by triethylamine, voltage 10 kV, capillary 20/27 cm (effective/total length), ID 50 μm, injection 3 s, temperature 25 °C.

substrate and product in pooled erythrocyte incubation mixture were not resolved due to high concentration of substrate. Separation of substrate and product in standard mixture improved with higher pH (data not shown). For further optimisation 80 mmol/L CAPS (pK = 10.4) titrated by triethylamine (pK = 11.0) to pH 10.0–11.4 was used. Product was not resolved from substrate in real samples at pHs ≤ 10.9 and was fully separated at pH 11.2 (Fig. 1). Significantly distorted peaks with low separation efficiency were obtained at pH 11.4. To improve separation substrate and product concentration of co-ion (CAPS, in the range of 30–180 mmol/L) and addition of methanol to background electrolyte (0–20%) were systematically varied. Finally concentration of 100 mmol/L of CAPS and 10% of methanol were found as optimum in respect to separation efficiency (102000 TP/m), separation time (3.0 min) and generated current (120 μA).

Final separation buffer consisted of CAPS (100 mmol/L) titrated by triethylamine to pH 11.2 with addition of 10% of methanol. Analyses were performed under 20 kV (740 V/cm). Under these conditions method was validated and real samples were measured. Migration time of standard of 6-mMP dissolved in water was 2.43 min (effective mobility of $9.3 \times 10^{-9}\ m^2\ V^{-1}\ s^{-1}$) while 6-mMP in real samples migrates in 2.95 min (effective mobility of $11.2 \times 10^{-9}\ m^2\ V^{-1}\ s^{-1}$) (Fig. 2). Differences of 18% in effective mobilities are supposedly caused by character of matrixes. Analyte was confirmed by spiking of standard of 6-mMP to erythrocytes after incubation and comparison of spectra of peaks.

3.2. Validation of the method

Limit of quantification of the method is 1.5 μmol/L (S/N = 6). The calibration curve expresses as correlation between concentration of 6-mMP (μmol/L) and corrected area of peak (arbitrary units) had a slope of 5531.7 (±88.9), an intercept of 542.8 (±419.1) and an r^2 value of 0.9979 (±0.0011) ($n = 5$). Recovery was determined

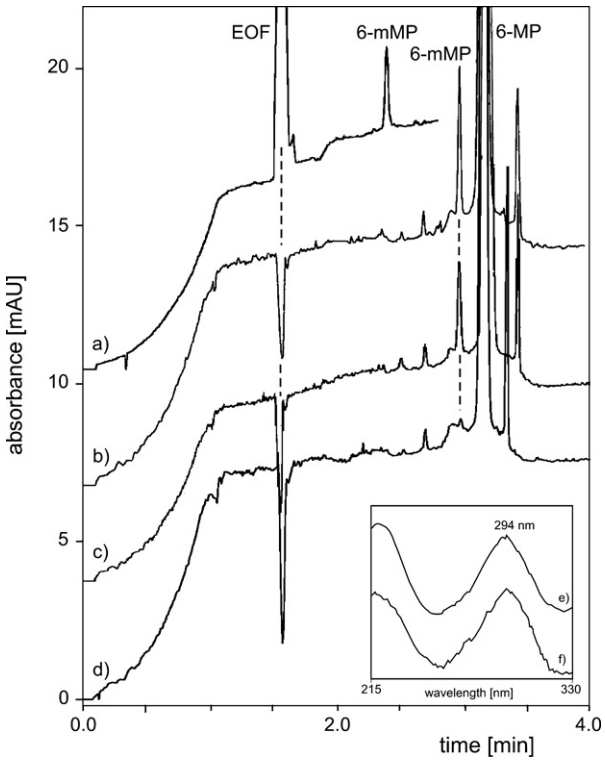

Fig. 2. Analysis of standard (a), real sample spiked by standard of 6-mMP (b), real sample with physiological enzyme activity of 40.7 nmol 6-mMP/(g Hb h) (c), and real sample with low activity of 9.4 nmol 6-mMP/(g Hb h) (d) of TPMT (main panel); spectra of 6-mMP standard (10 μmol/L) (e), and spectra of 6-mMP in real sample (f) (inset). Conditions: 100 mmol/L CAPS titrated by triethylamine, pH 11.2 with addition of methanol (9:1), voltage 20 kV, other conditions as Fig. 1.

in spiked erythrocyte lysates after back-extraction and was from 87.5% to 94.8%. Imprecision ($n = 10$) was 1.68% (within-day CV) and 2.53% (between-day CV) for 20 μmol/L addition of 6-mMP. Reproducibility of migration times measured using 10 samples of healthy volunteers was 0.46%, 0.79% and 1.42% for run-to-run, sample-to-sample and between-day measurements, respectively.

3.3. Analysis of TPMT activity in human erythrocytes

Using the developed method the TPMT erythrocyte activity in healthy 60 adult volunteers (age 36 ± 16 years, mean ± 2SD, 34 males, 26 females) was measured. Activities were in the range of 54.7 ± 12.3 nmol/(g Hb h) (mean ± 2SD) in adults. The observed data are in agreement with previously published data [9].

4. Conclusions

Screening for TPMT activity is important before starting treatment with thiopurine drugs, because TPMT deficiency can lead to severe, eventually fatal adverse reactions in patients treated with standard doses of the drugs. We developed capillary electrophoretic method for determination of TPMT enzyme activity in erythrocytes. The method is sensitive enough for screening of TPMT activity in human erythrocytes. No interferences were observed in all analyses of real samples. Total separation time including conditioning of capillary is less than 6 min. This approach represents fast and cost effective alternative to previously published procedures.

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